Urokinase Receptor Antagonists Inhibit Angiogenesis and Primary Tumor Growth in Syngeneic Mice

Hye Yeong Min, Laura V. Doyle, Charles R. Vitt, Catherine L. Zandonella, Jennifer R. Stratton-Thomas, Marc A. Shuman, and Steven Rosenberg

ABSTRACT

Urokinase plasminogen activator (uPA) and its receptor are key components of a cell surface proteolytic cascade used by tumor cells and capillary endothelial cells for basement membrane invasion, a process required for metastasis and angiogenesis. We have cloned, expressed, and purified the epidermal growth factor-like domain of murine uPA alone and fused it to the Fc portion of human IgG as high-affinity murine urokinase receptor antagonists. These molecules are potent inhibitors of murine urokinase binding to its receptor and inhibit angiogenesis in an in vitro model of capillary tube formation in fibrin gels. In vivo, basic fibroblast growth factor-induced neovascularization and B16 melanoma growth in syngeneic mice are also substantially suppressed by these molecules. Coupled with previous studies showing inhibition of metastasis, these findings suggest that urokinase receptor antagonists may be useful therapeutically as inhibitors of tumor progression.

INTRODUCTION

Angiogenesis, the formation of new capillaries from existing blood vessels, is essential for the growth of solid tumors beyond 3 mm³ in size (reviewed in Ref. 1). Recent studies have shown that tumor growth can be suppressed by blocking the action of tumor-expressed angiogenic factors, such as VEGF² (2, 3). Thus, antiangiogenic agents may be useful therapeutically as long-term tumoristic treatments, especially for preventing the growth of distant micrometastases of primary tumors.

During angiogenesis, endothelial cells need to divide, migrate, invade into the extracellular matrix, and form capillary structures. The process of cell invasion requires the production of active cell surface proteases. Among these proteases is uPA, the receptor of which, uPAR, is a key molecule in cell surface proteolysis of the extracellular matrix by tumor cells and capillary endothelial cells (4). Treatment of capillary endothelial cells with angiogenic factors such as bFGF and VEGF cause up-regulation of uPA (5) and uPAR (6, 7). Furthermore, endothelial cell migration is inhibited by a uPAR antagonist (8), and formation of capillary tubes by bFGF-treated microvascular endothelial cells is decreased with anti-uPA antibodies (9). We sought to determine directly whether an antagonist of the urokinase receptor could suppress angiogenesis and tumor growth in mice by inhibiting the cell surface localization of uPA. In this report, we show that uPAR antagonists inhibit tumor growth in an in vitro syngeneic model. Furthermore, we show that uPAR antagonists inhibit angiogenesis in both in vitro and in vivo models, suggesting that the inhibition of tumor growth by uPAR antagonists may be due to the inhibition of angiogenesis.

MATERIALS AND METHODS

Reagents. Fibrinogen (fraction 1) from bovine plasma and thrombin from human plasma were obtained from Sigma Chemical Co. (St. Louis, MO). Matrigel was purchased from Collaborative Biomedicals Products (Bedford, MA).

Cloning of muPAR1 and muPAR2. The cDNAs for both forms of the mouse urokinase receptor (10) were cloned by PCR using a mouse macrophage cDNA library as template (Clontech, Palo Alto, CA). The sense strand primer for muPAR2 had the following sequence: 5'-GACTGGATCCCCATGGGACGCGG-3', and antisense strand primer was 5'-GACCTTCTAGAGTCAAGTGGGCG-3'. The antisense primer was 5'-GACCTTCTAGAGTCAAGTGGGCG-3'. The PCR reaction consisted of 100 pmol of each primer in 100-µl volume with standard PCR buffer and 5 units of Taq polymerase. The reaction was carried out for 30 cycles of 1 min at 95°C, followed by annealing for 1 min at 55°C, and extension at 85°C for 2 min. Further extension was at 85°C for 10 min, followed by a 15°C soak cycle. A PCR product of approximately 1150 bp was gel isolated, cut with BamHI and XhoI, then ligated into the pcDNA1-Neo vector (Invitrogen, San Diego, CA). The ligation reaction was transformed into electrocompetent MC1061/P3 cells (Invitrogen), and plasmid DNA was prepared using a Qiagen miniprep column (Qiagen, Chatsworth, CA). The sequence of this plasmid DNA was verified to be identical to the published sequence of muPAR2 (10). To clone the muPAR1 cDNA, the segment of muPAR2 spanning the internal XhoI site to the XbaI site at the 3' end was replaced by the corresponding fragment from the muPAR1 cDNA. This fragment was obtained by PCR amplification using the mouse macrophage cDNA library as template and the following primers: sense, 5'-CCCTTACCTCCAGTGTCGGC-3'; and antisense, 5'-GACCTTCTAGAGTCAAGTGGGCG-3'. The PCR reaction conditions were as described above. The muPAR1 cDNA was transformed into electrocompetent MC1061/P3 cells, and plasmid DNA was prepared as described above. The sequence of the XhoI-XbaI fragment was verified to be correct. For transfer of the muPAR1 cDNA inserts into the baculovirus expression vector pAcC13, the BamHI-XhoI fragment was excised from pcDNA1-Neo/muPAR1 and was ligated together with the 3' half of the insert, in which the XhoI site at the 3' end had been replaced with a NotI site by PCR, into pAcC13 at the BamHI and NotI sites. The ligations were transformed into electrocompetent HB101 cells (Bio-Rad, Richmond, CA), overnight cultures were made from colonies, and plasmid DNA was prepared using Qiagen columns. The miniprep DNA was transfected into baculovirus for expression of muPAR1 in SF9 cells.

Cloning of Soluble Mouse uPA Receptor and Expression in Baculovirus. To express soluble mouse uPA receptor, the cDNA sequence of muPAR1 was truncated between the Ser-272 and Pro-273 (amino acids numbered from initiating methionine). A 9-amino acid epitope tag (glu tag) was added at the 3' end of the protein to aid purification. This construct was made by using PCR to incorporate the truncation site and the epitope tag as well as a NotI site at the 3' end of the fragment for cloning into the baculovirus vector pAcC13. The primers used were: sense, 5'-CCCTCCTCCAGGGCGCTTTTACCTCCAGTGGTCGG-3'; and antisense, 5'-CCCTCCTCCAGGGCGCTTTTACCTCCAGTGGTCGG-3'. This PCR fragment was ligated along with the BamHI-XhoI fragment of muPAR1 into pAcC13 vector cut with BamHI and NotI. The ligation reactions were transformed into electrocompetent HB101 cells, and plasmid DNA was prepared using a Qiagen mini-column. The sequence of the insert was confirmed to be correct. The soluble muPA receptor expressed in a 250-ml culture of baculovirus-infected SF9 cells, and the conditioned media were collected. Purification of the protein was achieved in one step by using an anti-glu antibody column (11). The SF9 supernatant was batch loaded onto 2 ml of PBS-equilibrated column at 4°C overnight. The beads were poured into a column and washed with 10 ml PBS. Soluble mouse
uPA and cloned into pAcC13 vector (14). The resulting plasmid, ml-48Ig, was ligated to the 3' end of a cDNA fragment encoding residues 1-48 of murine CH3 domains of the human IgGl gene (nucleotides 892-1859 Ref. 13) was under the conditions reported previously (12).

Protein was then eluted with a linear gradient to a final concentration of 2 M NaCl in the above buffer. Fractions containing ml-48 or ml-48glu were pooled and separated further via high-performance liquid chromatography column and washed with 50 mM succinic acid (pH 6.0). 50 mM NaCl buffer. 

Fractions containing monomeric protein from the sizing column were pooled and loaded onto an SP Sepharose fast flow (Pharmacia) in accordance with the National Association for Biomedical Research guide for the humane care and treatment of laboratory animals. C57BL6 mice weighing between 18-20 g received s.c. injections of 3.5 mg/ml fibrinogen in PBS. Thrombin (50 µl of 25 units/ml in cell culture media) was added and gently mixed. This mixture was transferred immediately to a 24-well plate and incubated for 5 min at 37°C to allow the fibrin gel to form. VEGF and bFGF (5 ng/ml each) were added to the cells along with uPAR antagonists in 1 ml of cell culture medium. The cells cultures in fibrin gel were incubated at 37°C with 5% CO₂ and photographed at days 1 and 4 at x5 using Kodak Tri-X film.

In Vivo Matrigel Angiogenesis Model. All animal experiments were done in accordance with the National Association for Biomedical Research guidelines for the humane care and treatment of laboratory animals. C57BL6 mice were grown for 96 h, then supernatants were collected for purification of ml-48/lg fusion protein containing bFGF with and without uPAR antagonists. The mixture consisted of 600 µl Matrigel and 195 µl additions. After 5 days, the animals were sacrificed and dissected to expose the Matrigel plugs for photography.

**Table I Quantiﬁcation of capillary tube formation in fibrin gels**

<table>
<thead>
<tr>
<th>% cells/field</th>
<th>Rounmd</th>
<th>Elongated, no branches</th>
<th>1-2 branches</th>
<th>&gt;2 branches</th>
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<tr>
<td>Day 1 media control</td>
<td>81.9</td>
<td>18.1</td>
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<td>0</td>
</tr>
<tr>
<td>Day 4 media control</td>
<td>86.1</td>
<td>13.8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Day 4 + 10 µM m1-48lg</td>
<td>12.8</td>
<td>10.5</td>
<td>12.8</td>
<td>63.9</td>
</tr>
<tr>
<td>Day 4 + 10 µM m1-48lg</td>
<td>9.0</td>
<td>13.5</td>
<td>9</td>
<td>51.5</td>
</tr>
<tr>
<td>Day 4 + 10 µM m1-48lg</td>
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<td>60.8</td>
<td>11.5</td>
<td>2.3</td>
</tr>
<tr>
<td>Day 4 + 3 µM m1-48lg</td>
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<td>46.2</td>
<td>13.7</td>
<td>0.9</td>
</tr>
<tr>
<td>Day 4 + 0.5 µM m1-48lg</td>
<td>26.9</td>
<td>34.8</td>
<td>13.5</td>
<td>24.7</td>
</tr>
<tr>
<td>Day 4 + 0.5 µM Fe</td>
<td>33.7</td>
<td>31.5</td>
<td>7.9</td>
<td>26.9</td>
</tr>
<tr>
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<td>14.7</td>
<td>10.7</td>
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<tr>
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<td>7.5</td>
<td>42.9</td>
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**Fig. 1. uPAR antagonist activity of ml-48/lg.** The data are plotted as the percentage of inhibition of total binding of [125I]-labeled ml-48glu by various concentrations of ml-48lg. Total cpm bound without competitor was 5203, and the nonspecific binding was 208 cpm. The IC₅₀ of ml-48lg was calculated to be 629 ± 56 psi by the EBDA program (Biosoft).

uPAR-glu was eluted using 8 ml PBS containing 50 µg/ml of EEEYMPME peptide and collecting 1-ml fractions. The fractions containing the majority of the protein were pooled. For biotinylation of soluble mouse uPAR-glu, 150 µg of protein were mixed with 4 ml of sodium borate buffer (pH 9) and concentrated on a Centricon 30 microcentrator (Amicon, Beverly, MA) down to 150 µl final volume. Biotin-XX-NHS (Calbiochem, San Diego, CA) was added to 100 µl protein at a 5:1 molar ratio of biotin:soluble mouse uPAR-glu. After incubating at room temperature for 1 h, the reaction was quenched with 150 µl of 1 M Tris-HCl (pH 7.5) and dialyzed overnight in a Pierce micro dialyzer against 500 ml of recirculated PBS.

**Cloning of m1-48 and m1-48glu.** Mouse uPA 1-48 with and without an added EEEYMPME epitope tag at the 3' end (ml-48glu-and ml-48, respectively) were cloned by performing PCR using murine uPA cDNA as template. The sense primer was 5'-CCGGAGATCTGTACTTGGAGCTCCT-

GGGCATGTATTCCTCTTCCTCTTTTGATGCATCTATC-3' for ml-

...dialyzer against 500 ml of recirculated PBS.

Fractions containing monomeric protein from the sizing column were pooled and loaded onto an SP Sepharose fast flow (Pharmacia) column and washed with 50 mM succinic acid (pH 6.0), 50 mM NaCl buffer. Protein was then eluted with a linear gradient to a final concentration of 2 M NaCl in the above buffer. Fractions containing m1-48 or m1-48glu were pooled and separated further via high-performance liquid chromatography under the conditions reported previously (12).

**Construction of Murine uPA 1-48lg cDNA and Purification of ml-48/lg Fusion Protein.** A BamHI-EcoRI fragment encoding the hinge, CH2 and CH3 domains of the human IgG1 gene (nucleotides 892-1859 Ref. 13) was excised from plasmid pBSig (a gift from G. Peltz, Syntex). This fragment was ligated to the 3' end of a cDNA fragment encoding residues 1-48 of murine uPA and cloned into pAcC13 vector (14). The resulting plasmid, ml-48lg/...
sites near the tumor injection site. In both tumor models, measurements were done using a caliper twice weekly starting at day 10 to calculate tumor volumes. Statistical analysis was done using the Mann-Whitney nonparametric U test (17).

RESULTS

uPAR Antagonist Activity of Murine uPA 1–48/Ig Fusion Protein. Urokinase plasminogen activator is a tripartite enzyme with an amino-terminal EGF-like receptor binding region. Because of the species specificity exhibited by human versus murine uPA for receptor binding (18), we expressed murine urokinase receptor antagonists comprising the EGF-like domain of mouse urokinase (amino acid residues 1–48) or this domain fused to the hinge, CH2 and CH3 domains of the human IgG1 heavy chain C-region (13) as a disulfide-linked dimer. The immunoglobulin fusion protein (m1–48Ig) inhibits binding of a radioactively labeled mouse uPA EGF-like domain with an IC50 of about 600 µM (Fig. 1), which is about 3-fold less potent than the yeast-derived murine uPA EGF-like domain itself.3 However, m1–48Ig has the advantage of a prolonged plasma half-life (>24 h in rats) compared to the mouse uPA EGF-like domain alone (<1 h in rats).4

Inhibition of in Vitro and in Vivo Angiogenesis by uPAR Antagonists. The formation of capillary tube-like networks by murine brain endothelial cells in fibrin gels was used as an in vitro model of angiogenesis (19). All of the cultures were treated with 5 ng/ml VEGF and 5 ng/ml bFGF throughout the experiment. At day 1, most of the cells were rounded and did not exhibit branching (Table 1). At day 4, the majority of cells in media without uPAR antagonists formed capillary-like structures having more than 2 branches (Fig. 2; Table 1). Addition of m1–48Ig to the media at 10 µM markedly inhibited capillary tube formation, as evidenced by a 30–50-fold decrease in the number elongated cells having more than two branches. Lower concentrations of m1–48Ig had correspondingly smaller effects, showing that the binding of uPA to its receptor is important for capillary tube formation in this model of in vitro angiogenesis. Human Fc fragment at 10 µM used as a control for the IgG portion of the m1–48Ig fusion protein, had very little effect on capillary tube formation.

To test directly whether urokinase receptor antagonists have antiangiogenic activity in vivo, we performed an experiment using s.c. injected Matrigel and bFGF to induce blood vessel growth in mice (20). After injection, the Matrigel mixture forms a plug that can be exposed to observe neovascularization. As shown in Fig. 3, at day 5, the mice receiving Matrigel containing 500 ng bFGF had extensive blood vessel growth. In contrast, addition of 400 µg m1–48Ig to the Matrigel/bFGF mixture significantly inhibited growth and invasion of new vessels, whereas the same amount of human 1–48Ig protein had a much diminished effect (data not shown). The mouse EGF-like domain alone at 250 µg inhibited neovascularization to a lesser degree than 400 µg m1–48Ig, perhaps due to more rapid in vivo clearance of the smaller protein.

Inhibition of B16 Tumor Growth by Murine uPAR Antagonists. We used a B16 melanoma model in syngeneic mice to investigate whether m1–48Ig could inhibit tumor growth. In one set of

Fig. 2. Inhibition of capillary tube formation in fibrin gels by m1–48Ig. Murine brain endothelial cells were plated in fibrin gels in 24-well tissue culture plates and observed for the formation of capillary-like structures at day 4 after plating. Additions to the media were: a, VEGF/bFGF; b, VEGF/bFGF + 10 µM m1–48Ig; c, VEGF/bFGF + 3 µM m1–48Ig; and d, VEGF/bFGF + 10 µM human Fc fragment. Bar in d, 400 µm.

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3 H. Y. Min, unpublished observations.
4 J. Gibbons, personal communication.
Fig. 3. Inhibition of bFGF-induced angiogenesis in mice by mL-481g. C57BL/6 mice received s.c. injections of 600 μl of a Matrigel mixture containing bFGF (23) with and without uPAR antagonists. After 5 days, the animals were sacrificed and dissected to expose the Matrigel plugs for photography. Each group contained six animals, and a representative animal from each group is shown. A, Matrigel containing 500 ng bFGF; B, Matrigel containing 500 ng bFGF and 400 μg mL-481g; C, Matrigel containing 500 ng bFGF and 250 μg mL-48. The amber color of the Matrigel plugs is due to bFGF, since the addition of mL-481g or mL-48 alone to Matrigel produced white plugs indistinguishable from the Matrigel plugs containing no additions (data not shown).

experiments (Fig. 4A), B16 melanoma cells were mixed with Matrigel and mL-481g before injection into a s.c. site in BDF, mice (21). By using Matrigel, which is liquid at cold temperatures but solidifies at body temperature, we were able to introduce the urokinase receptor antagonist in single administration. Mice treated with mL-481g were without measurable tumors for 17 days, whereas control mice developed tumors with an average volume >1000 mm³. By day 21, mL-481g-treated mice developed small tumors that continued to grow. This tumor growth suppression by mL-481g is not due to a direct effect of mL-481g on tumor cell growth, since the B16BL6 cell proliferation in vitro is unaffected by mL-481g. Thus, the substantial in vivo tumor inhibition observed is due to an indirect effect of mL-481g on tumor growth.

We next tested whether local injections of mL-481g around the site of tumor cell implantation could inhibit tumor growth when administered after tumor cell injection (Fig. 4B). One group of mice (n = 5)
RESULTS were statistically significant at $P < 0.05$ using the Mann-Whitney nonparametric $U$ test. In the same experiment, we also tested the effect of the recombinant mouse uPA EGF-like domain (m1–48) alone. Mice treated with 25 $\mu$g of m1–48 in daily injections had tumor volumes comparable to those treated daily with 200 $\mu$g m1–48Ig. Thus, the effect of m1–48Ig on tumor growth is not attributable to the human IgG portion of the molecule.

DISCUSSION

We have constructed protein ligands of the murine urokinase receptor that function as antagonists for plasminogen activation by virtue of their lack of catalytic activity and retention of receptor binding. The recent cloning of the murine urokinase receptor (10) and murine uPA enabled us to construct analogous molecules to those previously demonstrated to be effective receptor antagonists in the human system (12, 22, 23). These two molecules, m1–48 and m1–48Ig, bind to the murine uPAR with subnanomolar affinity, similar to that seen for the human ligands with the human receptor (12). In addition, we have constructed a variant of m1–48 with an epitope tag, which permits the efficient radiolabeling of this protein without loss of receptor binding activity. We have used these reagents to probe the role of urokinase receptor-dependent plasminogen activation in tumor growth and angiogenesis in mouse models. The results demonstrate that inhibition of uPA binding to uPAR can inhibit angiogenesis in in vitro and in vivo models. In addition, these inhibitors can suppress tumor growth in vivo, most likely by reducing tumor-induced neovascularization.

It has been suggested by a variety of studies that plasminogen activation is necessary for tumor angiogenesis and metastasis (reviewed in Ref. 24). In particular, uPA and its receptor, uPAR, are induced in capillary endothelial cells by a variety of pro-angiogenic factors, including bFGF and VEGF (5–7). During physiological angiogenesis, both uPA and PAI-1 are spatially and temporally regulated (25), and in transgenic mice which develop cutaneous melanomas, uPA expression is strikingly localized to the tips of capillary sprouts, where it is presumably involved in degradation of basement membrane prior to capillary sprouting (26). Both uPA and uPAR also are necessary for cell invasion and tumor cell metastasis in some systems (22), and uPA, uPAR, and PAI-1 are all independent prognostic indicators in human breast cancer (27, 28). Finally, it has been shown recently that uPA and PAI-1 levels correlate with microvessel counts and tumor invasion in primary invasive breast carcinomas (29).

This work represents the first direct demonstration that blockade of cell surface localization of urokinase can block both angiogenesis and tumor growth. Previous work has shown that metastasis can be blocked by anti-uPA antibodies (30–32), and that peptides or proteins that block uPA binding to the receptor can reduce metastasis. Crowley et al. (22) showed that expression of a catalytically inactive mutant uPA in human prostate cancer cells dramatically reduced metastasis in nude mice but had no effect on primary tumor growth. The lack of effect on tumor growth is likely due to the relatively lower affinity of human uPA binding to the murine receptor on the rodent endothelium.

The results described in this study as well as prior results showing urokinase receptor antagonists are effective inhibitors of tumor invasion and metastasis, suggest the potential of such molecules as anti-cancer agents targeting both angiogenesis and metastasis. The fact that high uPA and uPAR levels correlate with negative outcome in breast cancer (27, 28) and with disease progression in melanoma (33) supports the idea that cell surface uPA is important in cancer progression, and that inhibition of this process may be useful therapeutically. A paradox in this regard is that high PAI-1 levels are also a strong prognostic indicator in breast cancer (27). A possible explanation for this observation is that tumor cell invasion requires an appropriate
balance of proteases and inhibitors (34), or that PAI-1 expression is a surrogate marker for angiogenesis, since PAI-1 is largely expressed by endothelial cells (35).

Definition of the molecular basis of the inhibition of tumor growth by urokinase receptor antagonists will require further study, although several possible mechanisms can be envisioned: (a) endothelial cell degredation of basement membrane, a process necessary for capillary sprouting, may be directly inhibited by inhibition of plasminogen activation by receptor-bound urokinase; (b) angiogenesis could be inhibited indirectly by prevention of the activation or release from the extracellular matrix of pro-angiogenic factors such as VEGF or hepatocyte growth factor (36, 37) by uPA or plasmin; (c) the tumor cell could be targeted directly by uPAR antagonists, modulating the remodeling of extracellular matrix by tumor cells and thereby reducing tumor-associated angiogenesis. Another possible target could be the inhibition of cell surface uPA activity on macrophages (38). A recent study has shown that the levels of uPA, microvesSEL density, and macrophage content were correlated in primary invasive breast carcinomas (29). Finally, it has been shown recently that uPAR is a uPA-dependent receptor for vitronectin on monocytic cells (39). This observation, along with the fact that the amino-terminal fragment of uPA can promote cell migration by bovine endothelial cells (40), suggests that modification of endothelial or monocytic cell adhesion by uPAR antagonists could also play a role in the observations described here. The development of species-specific antagonists of the human and murine uPA receptors will enable us to dissect the stromal and tumor cell components of these phenomena in both syngeneic models and those using human tumors in immunodeficient mice.

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